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THE ROLE OF NEWLY DISCOVERED EXOTOXIN (S TOXIN) IN PSEUDOMONAS AERUGINOSA INFECTIONS

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Annual Report

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SUMMARY

A. Our long term goal is to determine the role of exotoxin S in humans infected with Pseudomonas aeruginosa and develop vaccines to reduce the morbidity and mortality associated with these infections. During the period of the project covered in this annual report (August 1, 1980-July 31, 1981) we have:

(1) Obtained direct evidence that exotoxin S is produced in humans infected with an S+ strain of P. aeruginosa (Sadoff, J.C. et al., manuscript in preparation).

(2) Begun to investigate the role of S in chronic pulmonary disease due to P. aeruginosa using a rat model (Thompson, M.R. et al., manuscript attached to progress report).

(3) Developed a Welek assay and a filter blot RIA for identifying mutants of P. aeruginosa altered in S production.

(4) Identified a proenzyme form of S which is 4,000 daltons larger than the enzymatically active form of S.

(5) Extended and completed a survey of clinical isolates to determine what percentage produces exotoxin S (Sokol et al., Infect. & Immun., in press (manuscript appended)).

(6) Continued to collaborate with Drs. C. Alving, A. Cross and J. Sadoff (Walter Reed Army Institute of Research) on Pseudomonas research and purified and supplied them with purified diphtheria toxin and Pseudomonas toxin A.

B. Publications resulting from this research.

1. Sokol, P.A., Iglewski, B.H., Hager, T.A., Sadoff, J.C., Cross, A.S., McManus, A., Farber, B.F. and Iglewski, W.J. Production of exoenzyme S by clinical isolates of Pseudomonas aeruginosa. Infect. & Immun. In Press (October issue, 1981). (Manuscript #1 in Appendix).

2. Thompson, M.R., Woods, D.E. and Iglewski, B.H. The role of Pseudomonas aeruginosa exoenzyme S in chronic lung infections in rats. To be submitted to Infect. and Immun. (Manuscript #2 in Appendix).

3. Friedman, R.L., Roerdiner, F., Iglewski, B.H. and Alving, C.R. Suppression of cytotoxicity of diphtheria toxin by monoclonal antibodies against phosphotidyl-inositol phosphate. J. Biophysics (In press, January, 1982). Copies available from Dr. C. Alving, Walter Reed Army Institute of Research, Washington, D.C. 20012).

4. Cross, A.S., Sadoff, J.C., Iglewski, B.H. and Sokol, P.A. Evidence for the role of toxin A in the pathogenesis of human infections with P. aeruginosa. J. Infect. Dis. 142:538, 1980. (Copies available from Dr. A. Cross, Walter Reed Army Institute of Research).

5. Sadoff, J.C., Iglewski, B.H., Cross, A.S. and Sokol, P.A. Evidence for the role of toxin S in the pathogenesis of human infections with P. aeruginosa. Manuscript in preparation (1980).

FOREWORD

In conducting the research described in this report, the investigator(s)
adhered to the "Guide for the Care and Use of Laboratory Animals,"
prepared by the Committee on Care and Use of Laboratory Animals of
the Institute of Laboratory Animal Resources, National Research Council
(THEW Publication No. (NIH) 78-23, Revised 1978).

During the course of this work the authors were greatly assisted by
Dr. Pamela Sokol, Mr. Jack Lile, and Ms. JoAnne Sherman. Their help is
deeply appreciated. Portions of this research were done in collaboration
with Drs. J.C. Sadoff, A. Cross and C. Alving, WRAIR, Washington, D.C. 20012.

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I.   Production of Exotoxin S in a Human Infected with a Toxin[+] Strain of P. aeruginosa.

A.   Introduction.

We previously examined a number of clinical isolates of P. aeruginosa for their ability to produce toxins S and/or A in vitro (1). Furthermore, patients recovering from infections with toxin A producing strains of P. aeruginosa have been shown to produce specific toxin A antibodies which provides indirect evidence that at least toxin A is produced in vivo in humans (2). This year we intensively studied a case of a fatal post operative P. aeruginosa bacteremia. The bacterial strain was found to produce large amounts of toxin S and small amounts of A in vitro. Both toxins S and A were found in the liver of this patient at postmortem examination. This provides direct evidence that toxins S and A are produced in humans during P. aeruginosa infections.

B.   Materials and Methods.

1.   Identification of Toxins A and S Produced In Vitro. The bacterial strain isolated from the patient was grown for 15 hr in dyalized trypticase soy broth (TSBD) with and without 10 mM nitrilotriacetic acid (NTA) as we previously described (3). Toxin A production was identified by a significant (greater than 20%) increase in adenosine diphophate ribosyl (ADPR) transferase activity in TSBD (no NTA) culture supernatants following preincubation with 4 M urea-1% dithiothreitol (DTT) (4). The enzyme reaction mixture (for toxin A assays) consisted of 10 µl of bacterial culture supernatants, 25 µl of wheat germ extract, 25 µl of buffer I (125 mM tris HCl, pH 7.0; 100 mM DTT) and 5 µl of ^{14}C-NAD (1).

Exotoxin S production was detected in supernatants from cultures grown in TSBD with 10 mM NTA utilizing, as a substrate, extracts of toxin A resistant polyoma transformed baby hampster kidney cells (PyBHKR) as previously described (1). The S enzyme reaction mixture consisted of 10 µl bacterial culture supernatant, 10 µl of buffer II (200 mM sodium acetate, pH 6.0), 10 µl PyBHKR extract and 5 µl ^{14}C-NAD.

The reaction mixtures for both toxins A and S assays were incubated at 25°C for 30 mins then terminated with 10% TCA and processed as previously described (4). The amount of toxins A and S produced were estimated from standard curves obtained utilizing pure toxins A and S.

2.   Detection of Toxins A and S in Human Tissues. Specific antisera to purified toxin A and toxin S were produced in rabbits as previously described (1). Reactivity of the specific antitoxins were determined by double diffusion precipitin analysis. Indirect direct immunofluorescent staining was performed on frozen liver sections. After water and phosphate buffered saline (PBS) rinses, the sections were exposed to 1/20 dilutions (in PBS) of a given rabbit antisera or normal rabbit sera (prebleds from the rabbits obtained prior to immunization). Following two PBS-washes, the sections were then treated with a 1/40 dilution of commercially obtained fluorescein-tagged goat anti-rabbit-gamma-globulin antiserum. The sections were examined for specific-fluorescence by epi-illumination microscopy.

C.   Results and Discussion.

The patient was a 76 year old man who had been in good health until 3 years previous to the present admission. At that time a diagnosis of transitional cell carcinoma of the bladder, stage A, grade I was made at transurethral prostatectomy. He received chemotherapy in addition to surgical extirpation of the malignancy. While being evaluated for possible recurrence of the malignancy an aortica abdominal aneurysm was discovered for which surgical correction was recommended. The repair

was done under thiopental and enflurane anesthesia and was complicated by hypotension secondary to bleeding. On the first postoperative day his distal lower extremities appeared cold. He returned to the operating room for removal under nitrous oxide anesthesia of thrombi from the popliteal, posterior and anterior tibial arteries. On the following day he was noted to have a termperature of $102^{8°}$F, purulent sputum, a urine output of 12 cc per hour, a urine culture with >10^5 organisms per ml Pseudomonas aeruginosa and Enterobacter aerogenes and bilirubin and SGOT of 280 units. On the third day after his initial procedure he was begun on hemodialysis. Jaundice became first evident on clinical examination and his bilirubin was 13.9 (10.9 direct). The fever continued while the leukocyte count showed a left shift, Doëhle bodies and toxic granulation. Blood cultures were negative. An above the knee amputation was performed on the next day under halothane anesthesia. A blood culture drawn for a temperature of 104°F was reported as positive for Pseudomonas aeruginosa on the next day (5th day after the initial procedure) and the patient received therapy with gentamicin to which this isolate was sensitive at a level of 1 µg/ml. At this time the bilirubin had risen to 19.5 which was attributed to the bacterial sepsis. Blood cultures from the 5th and 8th days (after the initial procedure) also grew Pseudomonas aeruginosa. From the 6th day following the aortic repair to his death on the 10th post-operative day the patient remained febrile, continued in shock despite dopamine therapy, was deeply jaundiced and continued on hemodialysis for the renal failure. On the 10th day after surgery the patient had seizures and a new pulmonary infiltrate. (Culture of the spinal fluid from a lumbar puncture done before his death grew Pseudomonas aeruginosa.) He died that same day.

Postmortem examination showed no evidence of malignancy involving vessel walls. There was a necrotizing pneumonitis as well as an extensive pyelonephritis with an acute inflammatory infiltrate characterized by necrosis and hemorrhage. The liver weighed 2,417 grams and the bile ducts were of average caliber. Histologic examination revealed an acute inflammatory infiltrate with granulocytes in the sinusoids and portal areas, but no hepatocellular destruction. The patient had 2.96 µg/ml antibody to the lipopolysaccharide of the infecting strain and 1.9 µg/ml antibody to toxin A in sera obtained at the time of the initial bacteremic episode. Toxin phenotypic analysis (see IB1 above) of the P. aeruginosa strain isolated from this patient indicated in vitro it produced small amounts of toxin A (<0.1 µg/ml) and relatively large amounts of toxin S (~5 µg/ml). Immunofluorescence with specific toxin S and toxin A antisera (see IB2 above) was employed to determine if these toxins were produced in vivo during this patient's infection with P. aeruginosa.

Positive fluorescence was obtained with frozen liver sections of this patient's lvier when reacted with either the specific toxin S or toxin A antiserum. Negative fluorescence was obtained when normal rabbit serum was substituted for anti-toxin A or antitoxin S or when liver sections obtained from uninfected kidney donors were subjected to the immunofluorescent procedure. These data provide the first direct evidence that toxin S and A are produced in humans infected with toxin producing strains of P. aeruignosa. Since there was no evidence of any biliary tract obstruction or hepatic cellular disease at postmortem examination our data suggests that perhaps toxin S and/or A may have caused metabolic alterations in liver cells resulting in the deep jaundice that was seen in this patient.

II.   Role of Exotoxin S in Chronic Pulmonary Disease of Rats Due to Pseudomonas aeruginosa.

     A.   Introduction.

An animal model of pulmonary P. aeruginosa infections, free of the pathological process of underlying disease, has been developed by Cash et al. (5).

Current studies in our laboratory have employed this model of chronic pulmonary infection with P. aeruginosa to determine the role of this organism and its products in the progression of pulmonary disease. The present studies were designed to assess the role of exotoxin S in chronic pulmonary infections due to P. aeruginosa.

B.   Materials and Methods.

1. Previously Described Methods. Many of the methods utilized in these sutdies were described in detail in past progress reports or in our publications. These include standard culture media (3); deferration, and iron determinations (6); bacterial strain (PS 388) characterization (3); bacterial maintenance (7); our standard ADP-ribosyl transferase assay (7) and immunofluorescence (described above in section IB).

2. Experimental Chronic Rat Lung Infection Model. Adult male, Sprague-Dawley rats, 200-250 g (Simonsen Labs, Gilroy, CA), were tracheostomized under ether anesthesia and 0.05 ml of 10^4 P. aeruginosa imbedded in agar beads placed in a distal bronchus via a bead-tipped, curved needle as described (5). At intervals after inoculation, groups of animals were exsanguinated by cardiac puncture under anesthesia. The lungs were then processed for histologic examination or were processed for bacterial quantitation as previously described (5).

C.   Results and discussion.

1. Bacteriology of Bead-Inoculated Lungs. Groups of rats were inoculated transtracheally with 10^4 P. aeruginosa strain 388 in agar beads. Four animals were sacrificed at 3, 9, and 30 days post-challenge and the lungs homogenized for bacterial quantitation. Table 1 shows the mean and standard error of the CFU of P. aeruginosa recovered from homogenized lung tissue at the 3 post-inoculation times. The number of bacteria recovered rose from an initial inoculum size of 10^4 to approximately 3×10^7 cfu/lung on day 3. On day 9, the number of recoverable organisms had fallen to approximately 2×10^6 cfu/lung. Approximately 3×10^6 organisms/lung were recovered on day 30, indicating that a chronic infection had been established.

Table 1. Bacterial Quantitation of Pseudomonas aeruginosa Strain 388 From Chronically Infected Rat Lungs

Days Post Inoculation	Colony Forming Units*
3	$3.2 \pm 0.4 \times 10^7$
9	$2.2 \pm 0.9 \times 10^6$
30	$3.3 \pm 2.8 \times 10^6$

2. Pathology of Bead-Inoculated Lungs. Three animals were sacrificed on each of the 3,9 and 30 day sampling times and examined for histopathological changes. Necrosis, inflammation, and pleural adhesions were observed upon gross visual inspection of lungs receiving beads containing P. aeruginosa strain 388. Firm, tan-white fibrous lesions were seen in the posterior lung zones at the 30 day sampling times as reported by Cash et al. (5).

Microscopic examination demonstrated both parenchymal and bronchial changes at all sampling times. Beads containing P. aeruginosa were found in airways and surrounded by acute inflammatory exudate. The bronchial wall was usually affected by chronic inflammatory changes and the parenchyma was often obscured by suppurative inflammation. Chronic granulomatous lesions that involved the surrounding parenchyma were elicited by beads reaching the respiratory bronchioles. Early findings of parenchymal involvement were demonstrated by increased numbers of alveolar macrophages and alveolar septal thickening. Alveolar septae away from the principal lesions were often congested with neutrophilic and mononuclear cells. Granulomatous lesions observed in sections of lesions at later stages, often contained a central bead invaded by neutrophils surrounded by organizing chronic inflammation.

3. Demonstration of In Vivo Exoenzyme S Production by Immunofluorescence. The antiserum used for the demonstration of in vivo exoenzyme S production was specific for exoenzyme S and had a neutralizing titer of 1/320. Employing this specific antiserum, positive fluorescence was demonstrated in lungs of animals infected with P. aeruginosa strain 388 and was associated with alveolar tissue. Negative fluorescence was obtained when normal rabbit serum was substituted for anti-exoenzyme S serum or when uninfected lungs were subjected to the immuno-fluorescent procedure. Additionally, when lungs from animals infected with P. aeruginosa strain PAO-1 (exoenzyme S negative) were examined, negative fluorescence was obtained.

4. Exoenzyme S Neutralizing Ability of Sera. Twenty-one rats infected with P. aeruginosa strain 388 (7 each from days 3, 9 and 30) were exsanguinated and the serum removed from whole blood. Following heat inactivation, these sera were tested for their ability to neutralize exoenzyme S activity. None of the sera tested were found to have any neutralizing ability against exoenzyme S beyond a ½ dilution. The immunocompetence of the animals was however demonstrated as all sera from PS 388 infected rats contained normal immunoglobulin levels as measured by radial immunodiffusion (8).

The data obtained from the present study are quite similar to those reported by Cash et al. (5). P. aeruginosa strain 388 organisms persisted throughout the entire 30 day study period and maintained a constant phenotype. Concommitant with the persistence of the organisms was the presence of exoenzyme S in the lungs of all animals at all time points. Since the exoenzyme S activity localized by immunofluorescence was associated with altered alveolar tissue, this suggests that exoenzyme S is involved with the pulmonary pathology associated wtih these P. aeruginosa lung infections.

As with the persistence data, the pulmonary pathology observed in animals infected with P. aeruginosa strain 388 was similar to that observed in previous studies (5). In contrast when we employed toxin A^+S^- strains of P. aeruginosa in this animal model the degree of infiltration was considerably decreased and alveolar septae remained intact. Furthermore, toxin A^-S^- strains while able to persist in rat lungs, produce only bronchial lesions with no apparent parenchymal involvement.

As the pathological changes in chronically infected rat lungs reported by Cash et al. (5) were found to closely mimic those reported in the present study, we examined the P. aeruginosa strain DG1 used by Cash in the original development of the model for in vitro exoenzyme S activity (1). In vitro levels of exoenzyme S activity produced by DG1 were found to be equivalent to those levels produced by PS 388. The present findings suggest that the patholgoical changes observed by Cash et al. (5) can in part be attributed to exoenzyme S.

The finding that sera obtained from P. aeruginosa strain 388 infected rats failed to neutralize exoenzyme S activity is an interesting one. To date, no data are available concerning the presence of antibodies to exoenzyme S in individuals with chronic pulmonary infections due to P. aeruginosa. Certainly, a study of this type, in view of the results obtained in the present study, is warranted. Recent observations (manuscript in preparation) have shown that exoenzyme S is associated with membrane vesicles on the bacterial cell surface similar to the LT toxin in Escherichia coli (9). The inability of the host to stimulate a systemic immune response in this localized, non-bacteremic infection model may be accounted for by the fact that the membrane material (e.g. lipid) may protect this antigen from being processed. Further studies on this phenomenon must be performed to test this hypothesis.

In summary, exoenzyme S appears to play a prominent role in the progressive pulmonary pathology associated with chronic P. aeruginosa pulmonary infection. We found that exoenzyme S is produced in vivo in areas of alveolar alterations. Additionally, the pathology associated with exoenzyme S producing strains is more closely related to the pathology observed in cystic fibrosis than that associated with non-exoenzyme S producing strains.

III. Production of Exoenzyme S.

A. Introduction.

Until recently the only method we had for monitoring production and purification of S involved measuring its ADP-ribosyl transferase activity (1,3,7). Thus we were inadvertently selecting conditions for production and purification of the enzymatically active form of S (hereafter referred to as exoenzyme S). Last year we succeeded in obtaining specific exoenzyme S antibody. We utilized this antibody to reinvestigate production of S in a defined medium and discovered that under certain conditions an enzymatically inactive form of S is produced. We believe this may be the toxic, proenzyme form of this protein. We also continued to investigate the production of S and have found it is associated with blebs (membrane vescicles).

B. Materials and Methods.

The bacterial strains, 388 and 388-6, enzymatic assays, defined and complex growth media, culture methods and specific S antisera have all bee described in detail in previous annual reports. S antigen was quantitated by single radial immunodiffusion as described (8).

C. Results and Discussion.

Strain 388-6 was grown in defined medium composed of KH_2PO_4 (15 mM), NH_4Cl (90 mM), monosodium glutamate (50 mM), Na succinate (110 mM), $MgSO_4$ (5 mM), glycerol (5%), $FeSO_4$ (18 µM) and NTA (10 mM) pH 7.0 at 32°C with vigorous shaking. Production of S was monitored both by radial immunodiffusion (8) and in the S specific ADP ribosyl transferase assay. Under these conditions approximately 5 ug of S is produced per ml of culture supernatant and it has very high enzymatic activity. When 388 or 388-6 is grown in the defined medium without NTA only very low ADP-ribosyl transferase activity is found in the culture supernatant (less than 5% of that in presence of NTA). Surprisingly, when S was quantitated by radial immunodiffusion we found that approximately the same amount of S antigen is produced in the absence of NTA (4.8 µg) as when NTA is added to the culture medium (5.0 µg).

The S proteins produced in the presence and absence of NTA were both precipitable with anti S antibody. These precipitates were dissolved in 0.5% NP-40 by heating at 100°C and electrophoresis on polyacrylamide gels in the presence of 0.25% tauro-deoxycholate (TDOC). Under these conditions the enzymatically active form of S has a molecular weight of 49,000 and the enzymatically inactive form of S has a molecular weight of 53,000 daltons. Most bacterial toxins which have an enzymatic activity exist in two forms; a proenzyme form which is toxic but enzymatically inactive and an enzymatically active form which is non-toxic. We believe the 53,000 dalton protein is the toxic proenzyme form of S.

Exoenzyme S is apparently secreted in blebs that bind to nitrocellulose filters and which form a clear gelatinous pellet when the culture supernatant is centrifuged at 100,000 x g for 18 hours.

We have studied these blebs by electron microscopy as follows: One drop of a bacterial suspension to be examined was placed on a 0.25% Formvar-coated copper grid and allowed to stand for 5 minutes. Excess fluid was removed with filter paper and a drop of distilled water was added to the grid for 30 seconds. This drop was removed with filter paper and a drop of 0.1% phosphotungstic acid in 0.1 M sodium cacodylate (pH 7.2) was placed on the grid for 30 seconds. Excess stain was removed with filter paper and the grid allowed to dry before examination by transmission electron microscopy. Grids were examined on a Phillips 301 Transmission Electron Microscope.

As shown in Figure 1, strain 388-6 appears to be surrounded by blebs. Interestingly S producing strains (either 388, 388-6 or DG-1) produced blebs even when grown in the absence of NTA whereas S⁻ strains (PA-103 and PAO-1) only produced blebs when grown in the presence of NTA.

Ferritin conjugated to antibody to exoenzyme S was employed to visualize exoenzyme S associated with the surface of the bacterial cell. The ferritin-antibody conjugates were produced as follows: One ml of a 6% ferritin solution was centrifuged at 100,000 x g for 2 hours, the supernatant discarded and replaced with an equal volume of 2% IgG prepared from the anti-exoenzyme S in 0.1 M sodium phosphate buffer, pH 6.8, in which the ferritin pellet was allowed to dissolve. A 1/10 volume of 0.4% glutaraldehyde was added and the mixture was allowed to react for 45 minutes at room temperature with constant agitation. The coupling reaction was stopped by the addition of 1 drop of 1.0 M $(NH_4)_2CO_3$, pH 8.8, and held 1 hour to ensure cessation of the reaction. The conjugate was centrifuged at 12,000 x g for 10 minutes to remove large aggregates and a portion of the conjugation was then applied to a K25-100 column (Pharmacia) of Bio-Gel A-5m 200-400 mesh (Bio-Rad) previously equilibrated with phosphate buffered saline. The column was washed with 2 x the volume of the column and 2.0 ml fractions collected. The ferritin concentration of the collected fractions was determined by measuring absorbance at 440 nm.

Grids bearing bacterial cells to be reacted with ferritin-antibody conjugates were washed twice with PBS. A drop of 5% (w/v) bovine serum albumin (BSA) was placed on the grids and the grids placed within a beaker containing wet filter paper for hydration for 5-10 minutes. After this time the excess BSA was "flicked off" the grid and replaced with a drop of conjugate. The grids were then returned to the beaker for 5-10 minute incubations. Excess conjugate was "flicked off" the grid and the grids were touched to three droplets of PBS on parafilm in rapid succession then washed 5 x one minute on five drops of PBS. This was followed by extensive water washings after which the grids were dried and examined with the electron microscope. As seen in Figure 2, the ferritin labeled S antibodies appear

to bind at the surface of the bacteria but not with the outer membrane of the bacterial. We believe the specific S antibody is reacting with the blebs which surround the bacterial cell. The LT toxin of E. coli has also been shown to be associated with membrane vescicles (9). Interestingly, using similar procedures employing specific toxin A antibodies, we have not been able to demonstrate toxin A associated with blebs regardless of the condition under which the toxin A[+] strains are grown.

Figure 1. Electron micrograph (final magnification - 20,000) of strain 388-6 grown in the presence of 10 mM NTA.
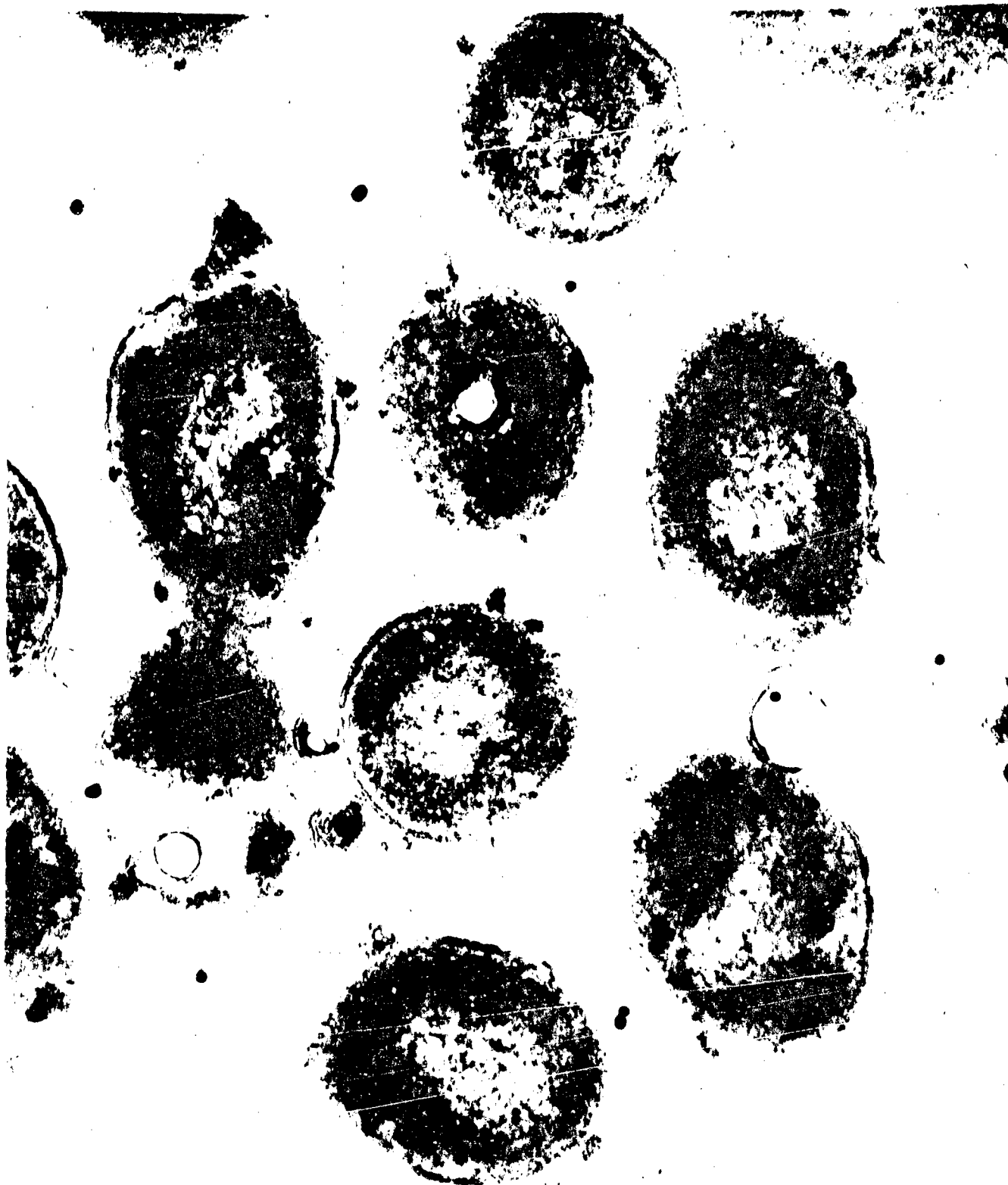
Figure 2. Electron micrograph of strain 388-6 grown without NTA and stained with ferritin conjugated S antibody. Final magnification 20,000X.

IV. Literature Cited.

1. Sokol, P.A., Iglewski, B.H., Hager, T.A., Sadoff, J.C., Cross, A.S., McManus, A., Farber, B.F. and Iglewski, W.J. Production of exoenzyme S by clinical isolates of Pseudomonas aeruginosa. Infection and Immunity, in press, October issue, 1981.

2. Cross, A.S., Sadoff, J.C., Iglewski, B.H. and Sokol, P.A. Evidence for the role of toxin A in the pathogenesis of human infection with Pseudomonas aeruginosa. J. Infect. Dis. 142:538, 1980.

3. Iglewski, B.H., Sadoff, J.C., Bjorn, M.J. and Maxwell, E.S. Pseudomonas aeruginosa exoenzyme S: an adenosine diphosphate ribosyl-transferase distinct from toxin A. Proc. Natl. Acad. Sci. USA 75:3211, 1978.

4. Vasil, M.L., Kabat, D. and Iglewski, B.H. Structure-activity relationships of an exotoxin of Pseudomonas aeruginosa. Infect. Immun. 16:353, 1977.

5. Cash, H.S., Woods, D.E., McCullough, B., Johanson, W.G. Jr. and Bass, J.A. A rat model of chronic respiratory infection with Pseudomonas aeruginosa. Am. Rev. Respir. Dis. 119:453, 1979.

6. Bjorn, M.J., Sokol, P.A. and Iglewski, B.H. Influence of iron on yields of extracellular products in Pseudomonas aeruginosa cultures. J. Bact. 138:193, 1979.

7. Bjorn, M.J., Pavlovskis, O.R., Thompson, M.R. and Iglewski, B.H. Production of exoenzyme S during Pseudomonas aeruginosa infections of burned mice. Infect. Immun. 24:837, 1979.

8. Mancini, G., Carbonara, A.O. and Hermans, J.F. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 2:235, 1965.

9. Grankema, H., Wensink, J., Gurinee, P.A.M., Jansen, W.H. and Witholt, B. Some characteristics of the outer membrane material released by growing enterotoxigenic Escherichia coli. Infect. Immun. 29:704-713, 1980.